Inability to Induce Hypertension in Normotensive Rat Expressing AT$_1$ Receptor Antisense

Alok S. Pachori, Hongwei Wang, Craig H. Gelband, Carlos M. Ferrario, Michael J. Katovich, Mohan K. Raizada

Abstract—Our previous studies have shown that neonatal delivery of angiotensin type 1 receptor antisense (AT$_1$R-AS) in a retroviral vector prevents spontaneously hypertensive rats from developing hypertension for life but has no effect on blood pressure (BP) in normotensive animals. Based on these results, we hypothesized that AT$_1$R-AS transduction in normotensive rats would protect them from developing experimental hypertension. The present study was designed to evaluate this hypothesis. A single intracardiac administration of AT$_1$R-AS by a retroviral-mediated delivery system (LNSV-AT$_1$R-AS) in 5-day-old normotensive Sprague-Dawley rats resulted in long-term expression of the AT$_1$R-AS without an effect on basal BP. However, angiotensin II (Ang II)–induced BP, dipsogenic responses, and renovascular contractility were significantly attenuated in the LNSV–AT$_1$R-AS–treated rats. Chronic infusion of low-dose Ang II (55 ng · kg$^{-1}$ · min$^{-1}$) in LNSV-alone–treated rats caused a modest increase in BP, profound increase in cardiac hypertrophy, and increased vascular contractility. In contrast, the LNSV-AT$_1$R-AS–treated rats were protected from developing these changes after Ang II infusion. These data establish that LNSV-AT$_1$R-AS pretreatment protects healthy rats from developing Ang II–dependent hypertension. (Circ Res. 2000;86:1167-1172.)

Key Words: angiotensin II ■ gene therapy ■ renin-angiotensin system ■ angiotensin type 1 receptor ■ hypertension

The renin-angiotensin system (RAS) plays an important role in human primary hypertension. Inhibition of angiotensin II (Ang II) formation or interaction with the angiotensin type 1 receptor (AT$_1$R) is important in controlling hypertension.1–3 Despite great success with traditional therapy, the cure for hypertension does not seem to be forthcoming. This may be because of many inherent disadvantages with treatment approach, including compliance, side effects, and lack of complete reversal of pathophysiological aspects of the disease.4 Previously, we explored the possibilities of genetic intervention in the expression of the RAS to control hypertension and circumvent these issues. This has proven to be highly successful at the conceptual level. Our studies demonstrated that a single intracardiac injection of a retroviral vector–containing AT$_1$R-antisense (AT$_1$R-AS) or angiotensin-converting enzyme (ACE)–antisense cDNA prevents the development of hypertension in the spontaneously hypertensive (SH) rat on a long-term basis.4–6 This sustained antihypertensive effect of antisense gene therapy was not accompanied by visual side effects and was associated with long-term expression of the AT$_1$R-AS transcript and decreased numbers of AT$_1$R in Ang II–targeted tissues.5 During the investigation, we noticed that despite a robust and comparable expression of AT$_1$R-AS, this strategy had no effect on the basal blood pressure (BP) of normotensive rats,4–6 leading us to hypothesize that the involvement of the RAS in the control of normal BP is limited. However, the contribution of the RAS is of greater significance during the development, establishment, and maintenance of hypertension. If our hypothesis is correct, we argued, then one should expect that the AT$_1$R–expressing normotensive rat would be protected from hypertension. The objective of the present study was to evaluate our hypothesis with chronic low-dose Ang II infusion in a rat model of hypertension.

Materials and Methods

Treatment of Rats With LNSV-AT$_1$R-AS

Pregnant female Sprague-Dawley (SD) rats were obtained from the breeding colony at Wake Forest University, Winston-Salem, NC. For the AT$_1$R-AS study, female offspring were removed from their mothers 5 days after birth and divided into 3 groups: saline control, retroviral (LNSV) vector control, and LNSV vector containing AT$_1$R,AT$_1$B receptor subtypes.9 Intracardial delivery of LNSV-AT$_1$R-AS, as previously described.3,6 AT$_1$R-AS (nucleotides –132 to +1128) was chosen because previous studies demonstrated that it blocked the functional aspects of both AT$_1$A and AT$_1$B receptor subtypes.9 Intracardial delivery of LNSV-AT$_1$R-AS, saline, or LNSV (viral control) was carried out in 5-day-old rats.4–6 At age 10 weeks, animals were ovariecotomized and fitted with ALZET 2004 minipumps (ALZET Corporation). Animals were lightly anesthetized with methoxyflurane (Metofane, Mallinckrodt Veterinary, Inc.), small incisions were made in the back, minipumps filled with physiological saline or Ang II were
inserted just below the skin between the shoulder blades, and the incisions were closed with wound clips. Animals were then returned to their individual cages. The pumps delivered 0.25 μL/h for 4 weeks for a final dose of Ang II of 55 ng·kg⁻¹·min⁻¹.

Physiological Measurements
Indirect BPs were monitored weekly for 4 weeks with the use of the tail-cuff method. At the end of this time period, the effect of Ang II on dipsogenic response was determined, as previously described. Water intake was expressed as milliliters consumed per kilogram of body weight.

Direct BP measurements were carried out in cannulated rats. The animals were anesthetized with a rodent cocktail containing ketamine (100 mg/mL) and xylazine (20 mg/mL), which was administered intramuscularly (0.7 mg/kg). Jugular vein and carotid artery were catheterized for direct BP measurements in free-moving, nonrestrained animals, with a pressure transducer coupled to a Digi-Med BP analyzer (Micro-Med). After a 30-minute equilibration period, the pressor response to Ang II (0.02 to 0.32 58 g/kg IV) was examined. The effect of losartan (10 mg/kg) on BP was also examined similarly. After the Ang II pressor response, cannula flushed with 0.9% physiological saline was administered intravenously as a control. Animals were then administered losartan intravenously, and the systolic BP was determined. Contractile responses of thoracic aorta and renal arterioles to Ang II were measured, as previously described.

Biochemical Measurements
A semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was used to measure the levels of AT,R-AS transcript and the Neo gene in various Ang II target tissues. Total RNA was subjected to a reverse-transcription reaction with the use of an AT,R sense primer (5'-CTCTCCTCATCTCCTGGTGG-3'). This procedure was followed by 33 cycles of PCR using both sense and antisense primer (5'-CCAGAAAGCCGTAGAACAGAGGG-3'). PCR products were analyzed by polyacrylamide gel electrophoresis. The sense primer used to detect the Neo gene expression was 5'-CGATCTGATCAAGAGACAGGAT-3' and the antisense primer was 5'-GCCACAGTGATGAACACAGAGG-3'.

RNase protection assay
RNase protection assay was used to detect viral AT,R-AS mRNA by synthesizing a sense probe using AT,R cDNA following manufacturer's protocol (Ambion). Genomic DNA was isolated and purified, and a PCR step was performed for generating a template for the synthesis of the labeled probe by adding a T7 promoter sequence to a 5' upstream primer in the following manner: 5’-GGATCTTATTACAGACTTCACTATCGGAGATTGGCCCCTTAACTCTT; 3’-CTGCGTAGAGGATGAAGCTCA. This step enabled the direct incorporation of the T7-promoter sequence into the template. This template was used in the in vitro reaction to synthesize the mRNA with 32P-CTP in the transcription reaction containing unlabeled 10 mmol/L ATP, 10 mmol/L UTP, 10 mmol/L GTP, and 100 μmol/L CTP and RNA polymerase. The reaction was incubated at 37°C for 1 hour and then treated with DNase I. The labeled probe was then gel purified and used within 24 hours. In the hybridization reaction, the labeled probe (4×10⁶ cpm) was hybridized with 30 μg of various RNAs. The RNA was precipitated with ammonium acetate followed by ethanol. Pelleted RNA was dissolved in hybridization buffer and incubated overnight at 42°C. The next day, the reaction was treated with 1:100 dilution of RNase A/RNase T1 mix in the digestion buffer. After centrifugation, the pellet was dissolved in the gel-loading buffer and ran on a 5% acrylamide gel. The gel was then exposed to film overnight at -80°C.

Statistics
All results are expressed as mean±SE. Indirect BP measurements were analyzed by repeated-measures ANOVA. Direct mean BPs were analyzed by ANOVA. Values of P≤0.05 were considered statistically significant. All experiments had n=7 per group, unless otherwise indicated.

Results
Characterization of AT,R-AS–Treated SD Rats
Administration of RNase-AT,R-AS viral particles to 5-day-old SD rats resulted in the expression of AT1R-AS transcript as early as 10 days after injection. This expression was maintained in various Ang II target tissues throughout the duration of the experiment because it was apparent at 100 days after injection. Representative RT-PCR data from a 14-week-old SD rat are presented in Figure 1. Expression of the AT1R-AS and the Neo gene transcript was found in the various peripheral tissues, as shown in Figures 1A and 1B, respectively. Expression of the AT1R-AS and Neo gene

Figure 1. Expression of AT1R-AS and Neo transcripts in the LNSV-AT1R-AS–treated rats. A, Expression of AT1R-AS transcript in 14-week-old ovariectomized SD rat treated with LNSV-AT1R-AS. Five-day-old SD female rats were injected with viral particles containing LNSV-AT1R-AS. Fourteen weeks after injection, various tissues were removed, and RNA was isolated and subjected to RT-PCR analysis. The ∼1.2-kb band corresponds to the AT1R-AS transcript. Positive control is from AT1R-AS–infected 3T3 cells, and negative control is from cells not infected with LNSV-AT1R-AS. B, Expression of Neo gene in the same tissues, as above; ∼650-bp band corresponds to Neo gene expression. C, Expression of Neo transcript in various brain areas of the ovariocytomized SD rat treated with LNSV-AT1R-AS from 2 different animals. D, Expression of AT1R-AS transcript in various brain areas of ovariocytomized SD rat treated with LNSV-AT1R-AS. Positive control is from LNSV-AT1R-AS–infected 3T3 cells, and negative control is the −RT control.
transcripts were also observed in several brain areas, including the cerebrum, hypothalamus, and brainstem (Figures 1C and 1D). These observations show that a single intracardiac administration of LNSV-AT1 R-AS in a 5-day-old rat causes high levels of AT 1 R-AS transduction in both the periphery and the brain throughout life. RNAse protection assay was used to confirm RT-PCR data. Figure 2 demonstrates a ∼350-bp band corresponding to a protected AT 1 R-AS transcript. 3T3 cells infected with the LNSV-AT 1 R-AS (positive control) demonstrated a robust expression of the transcript, whereas control cells did not show this band.

Despite the long-term expression of the AT1-R-AS, no significant difference was observed between basal BP in LNSV-AT1 R-AS–treated rats (105±2.0 mm Hg) and control rats (saline or LNSV, 109±1.0 mm Hg; Figure 3A). Previous studies have implicated a protective role of estrogens on the BP.10 –12 Additionally, the estrous cycle influences the responsiveness to Ang II. 13–15 Therefore, we ovariectomized female rats to eliminate such a protective effect and the cyclic response to Ang II. Figure 3B shows that ovariectomy in adulthood has no effect on basal BP in either LNSV-treated or LNSV-AT 1 R-AS–treated rats. Thus, all subsequent AT1-R-AS experiments were performed in ovariectomized rats.

Next, we studied the physiological consequence of AT1-R-AS expression. Ang II is a potent dipsogenic hormone, and a single subcutaneous dose of Ang II (150 μg/kg) in LNSV-treated control rats caused an average water intake of 11±1.5 mL · kg⁻¹ · h⁻¹ (n = 7) compared with an intake of 4.5±1.5 mL · kg⁻¹ · h⁻¹ after administration of saline. The drinking response to the same concentration of Ang II was 4.4±1.5 mL · kg⁻¹ · h⁻¹ (n = 7) in the LNSV-AT1 R-AS–treated rats, indicating a complete attenuation of the dipsogenic response by the antisense treatment (Figure 4). Amount of water intake was not different from the amount observed in the same animals after saline administration (1 μL/kg).

Figure 5 represents the effect of acute Ang II administration on changes in systolic BP. Increasing doses of Ang II caused a significantly greater change in systolic BP in LNSV-treated rats compared with LNSV-AT1 R-AS–treated rats, demonstrating the reduced response to acute peripheral Ang II. This difference was more pronounced at lower doses of Ang II than at higher doses. The in vitro vascular response demonstrated a similar attenuation to Ang II. Ang II caused a dose-dependent increase in contraction of both thoracic aorta and renal arterioles with a similar EC₅₀ of 43 nmol/L in the LNSV-treated and LNSV-AT1 R-AS–treated rats (Figure 6). However, the maximal contraction was decreased by 40% in

Figure 2. RNAse protection assay (RPA) for the detection of expression of AT1-R-AS in the LNSV-AT1 R-AS–treated rats. Expression of AT1-R-AS transcript in 14-week-old ovariectomized SD rat treated with LNSV-AT1 R-AS. Five-day-old SD female rats were injected with viral particles containing LNSV-AT1 R-AS. Fourteen weeks after injection, tissues were removed and RNA was isolated and subjected to RPA. Positive control is from LNSV-AT1 R-AS–infected 3T3 cells, and negative control is from cells not infected with the virus. Lane 1 shows 50-bp DNA ladder; lane 2, yeast mRNA (+RNAse) (β-actin probe); lane 3, yeast mRNA (−RNAse) (β-actin probe); lane 4, yeast mRNA (+RNAse) (AT1 sense probe); lane 5, yeast mRNA (−RNAse) (AT1 sense probe); lane 6, 3T3 (positive control); lane 7, 3T3 cells (negative control); lane 8, heart; lane 9, kidney; lane 10, adrenal; and lane 11, liver.

Figure 3. Effect of LNSV-AT1 R-AS treatment and ovariectomy (OVX) on indirect basal BP in 9-week-old SD rats. Five-day-old SD females were injected with LNSV control virus and LNSV-AT1 R-AS. At 7 weeks of age, all animals were ovariectomized. After a 1-week recovery period, BPs were measured by tail-cuff method. A, Effect of LNSV-AT1 R-AS treatment on basal BP of nonovariectomized rats. B, Effect of ovariectomy on LNSV-AT1 R-AS–treated rats. Data are mean ± SEM; n = 7.

Figure 4. Dipsogenic response to a bolus dose of Ang II in LNSV-AT1 R-AS–treated ovariectomized SD rats. Water intake was measured in control ovariectomized, LNSV-treated, and LNSV-AT1 R-AS–treated rats in response to saline and Ang II (150 μg/kg SC). Data are mean ± SEM; n = 7. *P < 0.001 vs saline control; **P < 0.001 vs LNSV.
Specificity of the Ang II effect is shown by the fact that KCl- or phenylephrine-induced contraction and acetylcholine-mediated relaxation were not different between the tissues from the LNSV-treated and LNSV-AT1 R-AS–treated rats (data not shown). Collectively, these observations demonstrate that AT1 R-AS expression in SD rats reduced Ang II–induced physiological responses without any influence on basal BP. This reduced physiological responsiveness may be associated with a decrease in the AT,R number, which has previously been shown in AT,R-AS–treated rats.6

**Effect of Chronic Ang II Infusion in LNSV-AT1 R-AS–Treated Rats**

Because acute Ang II–induced BP and dipsogenic responses were significantly attenuated with antisense treatment, we investigated whether Ang II–induced hypertension would also be affected in the LNSV-AT1 R-AS–treated rat. Figure 7 shows that 55 ng·kg⁻¹·min⁻¹ infusion of Ang II caused a gradual increase in BP, which reached a maximum of 128±5 mm Hg in 4 weeks in LNSV-treated rats (Figure 7A). However, the Ang II infusion failed to increase BP in LNSV-AT1 R-AS–treated rats, with BP similar to that observed in the ovariectomized control without the Ang II pump. A similar difference in the effect of Ang II was observed when direct BP was measured; ie, BP in LNSV-AT1 R-AS–treated rats was significantly lower than in LNSV-treated rats and controls (Figure 7B). Intravenous administration of losartan (10 mg/kg) in chronically Ang II–treated rats caused a 13±4 mm Hg decrease in BP in LNSV-treated rats, whereas the same dose of losartan resulted in a 6±2 mm Hg decrease in the BP of LNSV-AT1 R-AS–treated rats (Figure 7C).

Cardiac hypertrophy is a hallmark of hypertension. Ang II–infused LNSV-treated rats showed a 65% increase in the ratio of heart weight to body weight (HW/BW; Figure 8). This hypertrophy was significantly reduced in the LNSV-AT1 R-AS–treated rats. Ang II–induced contraction of the renal artery was also reduced in LNSV-AT1 R-AS–treated rats (Figure 6).
renal artery was increased in LNSV-treated rats after chronic Ang II infusion. This increase was attenuated in the LNSV-AT-R-AS–treated rats such that it was comparable to renal artery of control rats that were not infused with Ang II (data not shown). These observations demonstrate that, in addition to protection from high BP, there was a significant improvement in cardiac and renovascular responsiveness in the AT-R-AS–treated rats.

Discussion

The most significant finding of the present study is that pretreatment with antisense targeting the RAS protects normotensive rats from developing Ang II–dependent hypertension. As a result, the antisense gene therapy strategy proves useful in the treatment and prevention of hypertension. Our studies previously demonstrated that interruption in the expression of ACE or the AT,R prevents the SH rat from developing long-term hypertension. In addition, the antisense strategy transiently reversed hypertension in the adult SH rat once it was established. What is unique about the results of the present study is that targeting of the RAS by a single neonatal injection of the retroviral vector containing AT-R-AS had little or no effect on basal BP, other cardiovascular functions, or behavioral aspects in the normotensive rat despite a long-term, robust expression of the antisense transcript. However, it did protect normotensive rats from developing hypertension and associated cardiac and renovascular pathophysiology.

Ovariectomized SD female rats were used for most of this study to minimize the influence of well-studied involvement of sex steroids in BP and cardiovascular-system regulation. Our results demonstrate that in vivo pressor and dipsogenic and in vitro vascular responsiveness to exogenous Ang II were significantly attenuated in adult animals that were neonatally treated with single injections of AT-R-AS.

Chronic infusion of Ang II has been used as an animal model that seems to mimic human primary hypertension. Both are characterized by a gradual increase in BP and are associated with structural and pathophysiologival changes in various cardiovascular-relevant organs. Therefore, chronic Ang II infusion represents a valuable model to dissect the mechanisms underlying hypertension. It is well-known that Ang II has a dose-dependent effect on BP in the Ang II infusion model of hypertension. In our unpublished findings, we observed that a higher dose of Ang II (150 ng · kg⁻¹ · min⁻¹) results in an increase of ~45 mm Hg over 4 weeks. Published studies have also demonstrated a similar phenomenon, with infusion of a higher dose of Ang II for a much shorter duration resulting in a similar 45- to 60-mm Hg dose-dependent increase in BP. We decided to use a low subpressor dose for a longer time period to allow observation of any effects of the antisense in this model and to mimic as closely as possible the development of Ang II–dependent human hypertension. Ang II infusion resulted in a 20- to 30-mm Hg increase in BP from the start of infusion in ovariectomized SD females. This increase was prevented completely in ovariectomized SD females treated with AT-R-AS.

Losartan and ACE inhibitors have been shown to cause regression or prevention of cardiac hypertrophy. In the present study, single administration of a bolus of LNSV-AT-R-AS in a 5-day-old rat resulted in a significant level of AT-R-AS expression, as measured by both RT-PCR and RNAse protection assay in various Ang II target tissues, such as heart, adrenal, kidney, and liver, an observation consistent with our previous data. The AT-R-AS expression seems to be associated with an inability of Ang II to induce high BP, an increase in HW/BW ratio, and a renovascular response to Ang II. An interesting aspect of these results is that despite modest increase in BP, low-dose Ang II infusion caused a significant increase in cardiac hypertrophy. This increase was prevented completely by LNSV-AT-R-AS treatment. It is worth pointing out that the level of hypertrophy was much more pronounced in our study than in similar studies. This may be due to the prolonged (4-week) infusion of Ang II in our study. It is also tempting to suggest that changes in BP alone could not be responsible for such a severe increase in cardiac hypertrophy, and direct hypertrophic effects of Ang II in cardiac tissue remodeling may contribute to this observation. Evidence in support of this view includes the following: (1) the degree of increase in HW/BW ratio in the chronic Ang II-infused model is similar to the degree of increase observed in the SH rat, even though the SH rat expresses considerably higher BP; (2) subpressor doses of Ang II infusion cause a profound change in HW/BW ratio comparable to the change seen in the SH rat; and (3) AT,Rs are present in the cardiac tissue, and their blocking by losartan prevents hypertrophy. Therefore, it may be possible to separate BP effects from direct Ang II–induced effects on cardiac hypertrophy. These observations, taken together, give additional support to the notion that high BP and tissue remodeling, 2 hallmarks of hypertension, are independently controlled. The former may be a result of endocrine RAS, and the latter may be a result of regulation of the tissue RAS.
Chronic infusion of subpressor doses of Ang II has been shown to cause an increase in AT\textsubscript{1}Rs, a phenomenon termed autopotentiation effect.\textsuperscript{25,26} As a result, there is an increased sensitivity to Ang II on BP, which may be attributable to increase in the AT\textsubscript{1}Rs.\textsuperscript{25} Our studies demonstrate that AT\textsubscript{1},R-AS treatment completely prevents the autopotentiation effect. The mechanism of autopotentiation and its resistance in the AT\textsubscript{1},R-AS–treated rats remains unelucidated, but it may be related to a decrease in the levels of AT\textsubscript{1},Rs in Ang II target tissues.

The mechanisms of hypertension caused by chronic Ang II infusion are not completely understood. The increase in BP can be mediated by a direct action on the vascular smooth muscle to increase total peripheral resistance, actions on the kidney to promote sodium retention, and resistance changes that are mediated by central effects of Ang II. Several investigators have demonstrated that hypertension induced by the use of chronic low-dose Ang II may be mediated through the CNS pathway.\textsuperscript{27,28} Although our experiments were not designed to identify the precise mechanism of Ang II–dependent hypertension, this study demonstrates for the first time that a systemic administration of AT\textsubscript{1},R-AS by a retroviral vector results in its transduction into the brain. Lack of a tight blood-brain barrier in neonatal rats may explain this observation. Expression of the AT\textsubscript{1},R-AS transcript was detected in several brain areas, including the hypothalamus, which is known to regulate Ang II–induced dipsogenic response. The dipsogenic response was significantly attenuated in the AT\textsubscript{1},R-AS–treated animals, which may be due to a decrease in central AT\textsubscript{1}R number.

In conclusion, our studies indicate that pretreatment with the LNSV-AT\textsubscript{1},R-AS protects normotensive rats from developing Ang II–induced hypertension. In addition, our data demonstrate that viral vector can transport the AT\textsubscript{1},R-AS across the poorly defined blood-brain barrier in neonates for its robust and long-term expression in the brain. Thus, these studies provide additional support to our view that antisense gene therapy that targets the RAS is a conceptually sound strategy for the prevention and possible cure of hypertension.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL56912 and HL52189). A.S.P. is a predoctoral fellow of the American Heart Association Florida Affiliate.

References

9. Lu D, Yu K, Raizada MK. Retroviruses-mediated transfer of an angiotensin type I receptor (AT\textsubscript{1}R) antisense sequence decreases AT\textsubscript{1}Rs and angiotensin II action in astroglial and neuronal cells in primary cultures from the brain. Proc Natl Acad Sci U S A. 1995;92:2914–2918.
Inability to Induce Hypertension in Normotensive Rat Expressing AT₁ Receptor Antisense
Alok S. Pachori, Hongwei Wang, Craig H. Gelband, Carlos M. Ferrario, Michael J. Katovich
and Mohan K. Raizada

_Circ Res._ 2000;86:1167-1172
doi: 10.1161/01.RES.86.11.1167

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/86/11/1167

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/